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Analysis of gene expression associated with cold acclimation in blueberry floral buds using expressed sequence tags

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Abstract

To gain a better understanding of changes in gene expression associated with cold acclimation in the woody perennial blueberry (*Vaccinium* spp.), a genomics approach based on the analysis of expressed sequence tags (ESTs) was undertaken. Two cDNA libraries were constructed using RNA from cold acclimated and non-acclimated floral buds of the blueberry cultivar Bluecrop and about 600 5′-end ESTs were generated from each of the libraries. About 100 3′-end ESTs were generated from the cold acclimated library as well. Putative functions were assigned to 57% of the cDNAs that yielded high quality sequences based on homology to other genes/ESTs from Genbank, and these were classified into 14 functional categories. From a contig analysis, which clustered sequences derived from the same or very similar genes, 430 and 483 unique transcripts were identified from the cold acclimated and non-acclimated libraries, respectively. Of the total unique transcripts, only 4.3% were shared between the libraries, suggesting marked differences in the genes expressed under the two conditions. The most highly abundant cDNAs that were picked many more times from one library than from the other were identified as representing potentially differentially expressed transcripts. Northern analyses were performed to examine expression of eight selected transcripts and seven of these were confirmed to be preferentially expressed under either cold acclimating or non-acclimating conditions. Only one of the seven transcripts, encoding a dehydrin, had been found previously to be up-regulated during cold acclimation of blueberry. This study demonstrates that analysis of ESTs is an effective strategy to identify candidate cold acclimation-responsive transcripts in blueberry.

Keywords: Cold hardiness; Cold tolerance; Dehydrin; Freezing tolerance; Genomics; *Vaccinium*

1. Introduction

Blueberry (*Vaccinium* spp.) is an important small fruit crop rich in antioxidants, usually grown in acidic and imperfectly drained soils that would otherwise be considered unfit for agricultural production [1]. The United States is the world's largest producer of blueberries, but there is a need to develop more winter hardy and spring frost-tolerant cultivars of this woody perennial [2]. Genetic evidence from numerous plants, including woody perennials, indicates that cold hardiness is a quantitative trait [3–8]. Considerable molecular evidence indicates that development of cold hardiness or cold acclimation is a complex phenomenon involving changes in gene expression that result in the alteration in metabolism and composition of lipids, proteins, and carbohydrates [9–11]. Genes induced during cold stress encode several different classes of gene products: enzymes required for the biosynthesis of osmoprotectants; lipid desaturases for maintaining membrane fluidity; protective proteins such as antifreeze proteins, dehydrins, chaperones, and mRNA-binding proteins; proteins involved in protein turnover including ubiquitin, ubiquitin-associated proteins, and other proteases; detoxification proteins; and proteins involved in signal transduction such as transcription factors, protein kinases, and phospholipase C [10–13].

Our laboratory has been using molecular, genetic, and physiological approaches to better understand cold hardiness in blueberry. Far less cold hardiness research has been carried out on woody perennials than on herbaceous

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annuals like *Arabidopsis*. We have shown that 65, 60, and 14 kDa dehydrins accumulate in cold acclimated floral buds of several *Vaccinium* cultivars and found good correlation between levels of dehydrins and cold hardiness levels [14]. A full-length 2.0 kb cDNA clone encoding the 60 kDa dehydrin has been isolated from a cDNA library constructed from cold acclimated floral bud RNA [15]. In addition, molecular markers have been used to construct genetic linkage maps of blueberry populations suitable for identifying quantitative trait loci (QTLs) for cold hardiness [16,17].

Expressed sequence tags (ESTs) are generated by single-pass sequencing of randomly picked cDNA clones. With the development of high-throughput DNA sequencing technology, EST analysis has become a rapid and relatively inexpensive way to identify genes, proteins, and metabolic pathways through homology with other sequences in Genbank. Analysis of ESTs can provide an overall picture of transcripts involved in organ or tissue development, as has been done with castor seeds [18], guard cells of *Brassica campestris* [19], xylem tissues of *Pinus taeda* [20], root hairs and nodules of *Medicago truncatula* [21,22], petal protoplasts of petunia [23], rose petals [24], and immature flower buds of *Lotus japonicus* [25]. ESTs generated from two or more cDNA libraries can help to identify transcripts preferentially expressed under various conditions, at different stages of development, or in different organs. In poplar, transcripts involved in wood formation have been identified by comparing ESTs generated from cambial meristem and xylem cDNA libraries [26]. In kelps, genes specific to developmental stages have been identified by comparing ESTs from sporophytic and gametophytic cDNA libraries [27]; and in rye, aluminum stress-related transcripts were identified by comparing ESTs from aluminum stressed and unstressed cDNA libraries [28]. Furthermore, by combining ESTs or other DNA sequencing results with microarray analysis, it has become possible to examine expression of thousands of genes at the RNA level at one time. In this way, transcript levels of ~8000 genes were examined after transferring *Arabidopsis* plants from warm to cold temperature, and 306 of these genes identified as being cold responsive [13].

In order to identify additional genes potentially involved in cold acclimation in blueberry, we have generated ~600 5′-end ESTs from each of two cDNA libraries developed from cold acclimated (CA) and non-acclimated (NA) floral buds of *V. corymbosum* L. cultivar Bluecrop and another ~100 3′-end ESTs from the CA buds. These ~1300 ESTs represent the first publicly available EST database for blueberry. Putative gene identities were assigned by homology to sequences in Genbank. Clones were categorized according to putative functions and the types of genes expressed under non-acclimating and cold acclimating conditions were compared.

2. Materials and methods

2.1. Construction of cDNA libraries and isolation of plasmid DNA

The CA cDNA library was constructed previously in the Uni-ZAP^{TM} unidirectional λcloning vector (Stratagene, La Jolla, CA, USA) as described in Levi et al. [15] using RNA from floral buds of field-grown plants of the highbush blueberry cultivar Bluecrop (*V. corymbosum* L.) that had accumulated 655 chill units. One chill unit equaled 1 h of exposure to temperatures between 0 and 7 °C. This corresponded to a collection time in mid-December and a cold hardiness level, expressed as lethal temperature$_{50}$ (LT_{50}) or temperature that kills 50% of the floral buds in a controlled freeze–thaw test, of −27 °C [14]. The estimated titer for the unamplified CA library was 7.2×10^5 pfu ml^{-1}.

For preparation of the NA cDNA library, floral buds from the same field-grown Bluecrop plants used for the CA cDNA library but that had accumulated 0 chill units were collected at the first of October. This corresponded to a cold hardiness level or LT_{50} of −10 °C [14]. Total RNA was extracted from floral buds using the RNeasy® plant mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol with some modifications. Approximately 250 mg tissue samples were ground to a fine powder with liquid nitrogen using a mortar and pestle. Ten milliliters of 'RLT' lysis buffer containing 50 μl of β-mercaptoethanol and 125 mg polyvinylpyrrolidone were added to the frozen powder, mixed, and allowed to stand at room temperature until the homogenate completely thawed. One milliliter aliquots of the homogenate were brought to 2% (w/v) sarkosyl and the tubes were placed on their sides for 10 min at room temperature, at which point the protocol was continued as per the manufacturer's instructions. Homogenates were treated with 30 units of RNase-free DNase (Qiagen, catalog no. 79254) for 15 min at room temperature prior to elution of the RNA from the RNeasy® spin columns. Total RNA was provided to Stratagene for purification of poly-(A^+) RNA and construction of a custom cDNA library using the Uni-ZAP^{TM} vector. The estimated titer for the unamplified NA library was 2.3×10^6 pfu ml^{-1}.

To convert phage clones to plasmid clones, mass excision of aliquots of the libraries was performed according to instructions provided by Stratagene. Plasmid DNAs to be used as templates for sequencing reactions were isolated using either the Wizard® *Plus* SV miniprep DNA purification system (Promega, Madison, WI, USA) or the REAL Prep-96 plasmid kit (Qiagen). Plasmid DNA was quantified spectrophotometrically, then digested with *Eco*RI and *Xho*I (New England BioLabs Inc., Beverly, MA, USA) to determine insert size. Insert size was estimated using the 1 kb Plus DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA) on 1% agarose gels.

2.2. DNA sequencing and analysis of sequence data

Single-pass nucleotide sequencing of recombinant plasmid DNAs was performed using Big Dye Terminator sequencing chemistry (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) either by us using an ABI PRISM™ 310 Genetic Analyzer or by the University of Maryland, Center for Agricultural Biotechnology–DNA Sequencing Facility (College Park, MD, USA). From the CA library, 633 clones were sequenced. Of these, 112 clones were sequenced from both the 5′ and 3′ ends and 521 clones were sequenced from the 5′ ends only. From the NA library, 613 clones were sequenced from the 5′ ends.

DNA sequences were trimmed of vector sequence manually or using the software package 'Lasergene' (DNASTAR Inc., Madison, WI, USA). Individual ESTs were translated into all six possible reading frames using the Baylor College of Medicine Search Launcher: Sequence Utilities website (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) to identify the most likely open reading frame and putative translational start sites. Sequences were then compared with the National Center for Biotechnology Information (NCBI) non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTX algorithm [29] and default parameters. Sequences that had no significant similarity with sequences in the protein database were compared with the nucleotide database using BLASTN and default parameters.

Individual ESTs were assembled into contigs using 'Lasergene', with parameters optimized for ESTs rather than for genomic clones. Consensus sequences from the contig analysis were also compared with the non-redundant protein database using BLASTX. The highest BLAST scores from either the individual ESTs or contigs were used to assign putative identities to the clones. Only the sequences with BLASTX or BLASTN scores >100 were considered significant [30–32].

ESTs with putative identities were classified into 14 functional groups and then into subgroups within each of these basic groups based on the catalog established for grape, *Vitis vinifera* [33], except that a category for plant hormone-related proteins (those proteins involved in hormone metabolism, hormone receptors, and hormone-induced and hormone-repressed proteins) was added. The assignments into the different categories were based on the authors' knowledge of biochemistry, plant physiology, and plant molecular biology, by reference to the BioCyc–MetaCyc: Encyclopedia of Metabolic Pathways website (http://www.MetaCyc.org/), and by searching related abstracts in PubMed (http://www.ncbi.nlm.nih.gov/PubMed/).

2.3. RNA extraction and Northern blots

RNA for Northern blot analysis was extracted from Bluecrop floral buds that had accumulated 0 (collected 10/2/02), 400 (12/4/02), 800 (1/8/03), and 1200 (2/26/03) chill units. RNA was extracted from ∼600 mg frozen samples using the 'hot borate' protocol outlined by Wilkins and Smart [34]. Total RNA (5 μg per lane) from each time point was separated on 1% agarose/formaldehyde gels, visualized and photographed to confirm good quality and that equal amounts were loaded, and blotted onto Brightstar-Plus™ nylon membranes (Ambion, Austin, TX, USA) by capillary transfer using the NorthernMax™ blotting and hybridization kit (Ambion). RNA was bound to the membranes by UV crosslinking.

For preparation of DNA probes, cDNA inserts were amplified from plasmid clones of interest using T7 forward and T3 reverse primers. PCR products were quantified by electrophoresing through 2% agarose gels with known concentrations of Low DNA Mass™ ladder (Invitrogen Life Technologies). About 25 ng of each cDNA insert was ^{32}P-labeled to a specific activity of $1.0–2.0 \times 10^8$ cpm μg^{-1} by random priming using the Megaprime™ DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were prehybridized with ULTRAhyb buffer from the NorthernMax™ kit for 30 min and hybridized overnight at 42 °C. The following day, blots were washed twice (15 min, 42 °C) with Low Stringency wash solution from the NorthernMax™ kit and then exposed to X-ray film (Sterling Bioworld, Dublin, OH, USA) with intensifying screens at −80 °C.

3. Results and discussion

3.1. Quality of the libraries and sequences

Floral buds from CA and NA field plants of the cultivar Bluecrop were used for construction of the CA and NA libraries. There were several reasons for choosing field plants for these studies over cold-treated greenhouse plants. First, the purpose of this research was to identify genes potentially involved not just in cold stress but in cold acclimation and we have used caution in our description and interpretation of this research to use the words "cold acclimation" and not "cold stress". In woody perennials of the temperate zone, cold acclimation is triggered by several environmental cues, not only low temperatures, and is generally considered a two-step process [35]. The first stage is induced by short photoperiod and the timing and speed of acclimation can be affected by other factors such as available moisture. The second stage is induced by low temperature. Therefore, field plants have the advantage in this type of study over cold-treated greenhouse plants of acclimating to cold under natural conditions, to progressively shorter photoperiods and colder temperatures. Second, for construction of the CA library, we wanted maximally winter hardy plants. Cold-treated greenhouse plants cannot achieve the maximum level of cold hardiness reached by field plants. Floral buds of Bluecrop, in particular, reach a maximum cold hardiness

Table 1
Quality of cDNA libraries and DNA sequences

	Cold acclimated	Non-acclimated
Number of clones picked	633	613
Average insert size (bp)	1394	970
Percent clones containing translational start site	18	12
Number of clones yielding high quality sequences		
5′ end only	497	587
5′ and 3′ end	111	
3′ end only	1	
Percent high quality sequences	96	96
Average length of sequences[a] (bases)	615	609

[a] Following removal of vector sequence.

level or LT_{50} of about −24 °C under cold room conditions (4 °C and short photoperiod) and a maximum cold hardiness level of −27 °C under field conditions [14,36].

The quality of the CA and NA libraries and DNA sequences, as assessed by average insert size, percent of clones containing a translational start site, percent of sequences with <2% Ns, average length of sequences, etc., is summarized in Table 1. To verify the presence and determine the sizes of cDNA inserts, plasmid DNA from each clone was digested with restriction enzymes, *Eco*RI and *Xho*I, and fractionated on agarose gels. The CA and NA libraries appeared to be of reasonable quality having average insert sizes of nearly 1.4 and 1.0 kb, respectively. Single-pass sequencing of the inserts and translation into all possible reading frames showed that 18% of the clones from the CA library and 12% from the NA library were full length. From BLAST results, about 2% of the clones from the CA library were found to encode rRNA, while the NA library did not have any rRNA clones. The small differences in average insert size and percent contaminating rRNA were probably due to the different RNA extraction protocols used for constructing the two libraries.

For comparison of ESTs between the two libraries, we obtained 5′-end sequences from cDNA clones from the CA and NA libraries. For use in a separate research project to develop expressed sequence tag-polymerase chain reaction (EST-PCR) markers for DNA fingerprinting and mapping in blueberry [37], we also sequenced from the 3′ ends of ∼100 clones from the CA library. Ninety-six percent of the clones sequenced from each library yielded high quality (<2% Ns) single-pass sequences. Overall, from the CA library, 720 high quality sequences were obtained from 609 clones (497 clones with 5′-end sequences only, 111 clones with sequences from both 5′ and 3′ ends, and 1 clone with 3′-end sequence only). From the NA library, 587 high quality sequences (from the 5′ end only) were obtained. The average read-length, after trimming vector sequences, was 615 bases for the CA library and 609 bases for the NA library. The ESTs were deposited into Genbank and assigned consecutive accession numbers beginning with CF810419.

3.2. Contig assembly

The software package ‘Lasergene’ was used to assemble the ESTs from each library into contigs or clusters based on the presence of overlapping, identical, or similar sequences. The ‘Lasergene’ program was run with default threshold settings for clustering sequences with at least 80% similarity or with 12 or more overlapping consecutive bases. When 5′ and 3′ sequences for each clone did not overlap, these sequences were placed within the same contig manually. The ESTs from the CA library yielded 430 contigs comprised of from 1 to 16 sequences. The ESTs from the NA library yielded 483 contigs and each contig included from 1 to 7 sequences. The number of contigs from each library is probably a slight overestimation of the number of distinct transcripts examined. This overestimation is due to sequencing from the 5′ ends, which can result in sequences from cDNAs that represent the same transcripts not being assembled into the same contig because their 5′ ends are truncated sufficiently to prevent overlap. The average length of the contigs from the CA library was 673 and 590 bp from the NA library. There were 266 and 353 singleton contigs (having only one EST) from the CA and NA libraries, respectively. This corresponds to a redundancy rate, chance that a new sequence will already be represented in the data set, of 56% [(609 − 266)/609] for the CA library and 40% [(587 − 353)/587] for the NA library. Therefore, the redundancy is slightly lower in the NA library than the CA library, indicating a more diverse array of expressed genes under non-acclimating conditions than under cold acclimating conditions.

Contig analysis was also carried out after combining all the ESTs from both libraries. In this case, 875 contigs were formed; of these, 373 contigs included sequences from the CA library only, 464 contigs included sequences from the NA library only, and 38 contigs included sequences from both libraries. Therefore, approximately 9% (38/430) of the distinct transcripts from the CA library were also represented in the clones sampled from the NA library; and, vice-versa, approximately 8% (38/483) of the distinct transcripts from the NA library were represented in the clones sampled from the CA library. To put it another way, 4.3% (38/875) of the total distinct transcripts were shared between the libraries. This may be an underestimate of the shared transcripts, however, for the same reason described above, because sequencing from the 5′ ends could result in some sequences from cDNAs that represent the same transcripts but do not overlap.

3.3. Overview of BLAST results

Slightly over half of the ESTs from both cDNA libraries could be assigned putative functions on the basis of sequence similarity to genes or proteins of known function in Genbank. Of the 609 clones from the CA library and the 587 clones from the NA library that yielded high quality sequences, 345 (57%) and 334 (57%), respectively, were

Table 2
Most abundant cDNAs from the cold acclimated library

Putative gene identification	# cDNAs[a]
Senescence-associated protein/putative monooxygenase	10 (0 in NA)[b]
Dehydrin	10 (9 in NA)
F1 ATPase subunit alpha	5 (5 in NA)
DNA J heat shock protein	4 (2 in NA)
Early light-inducible protein	4 (0 in NA)
Beta amylase	4 (0 in NA)
Mei2-like cell cycle protein	4 (0 in NA)

[a] Number of times a particular cDNA was picked from the CA library.
[b] In parentheses is the number of times a particular cDNA was picked from the NA library for comparison.

assigned putative identities. The sequences from the remaining clones showed either significant similarity to protein or DNA sequences that were of unknown function (27% for both libraries–166/609 for the CA library and 157/587 for the NA library) or no significant similarity to any other sequences in the databases (16% for both libraries—95/609 for the CA library and 96/587 for the NA library).

3.4. Highly abundant cDNAs/transcripts from each library

The type and relative abundance of clones in a primary cDNA library should represent the pattern of expressed genes in the organ or tissue from which the cDNA library was derived. Of those clones that could be identified from Genbank searches, the most highly abundant ones (clones that were picked at random four or more times) from the CA and NA cDNA libraries are presented in Tables 2 and 3. For comparison, the number of times the clones were picked from the other library is shown in parentheses. Those that were picked many more times from one library than from the other library potentially identify differentially expressed transcripts.

The clones that were picked the most number of times (10 times) from the CA library encode senescence-associated/putative monooxygenase and dehydrin proteins (Table 2). The senescence-associated cDNA was not picked even once from the NA library, strongly suggesting that it represents a transcript associated with cold acclimation. Homologous genes have been found to be associated with degradative metabolism in senescing petals of daylily [38], down-regulated during post-harvest storage of snow pea pods [39] and, from microarray analyses, up-regulated in *Arabidopsis* during cold stress [13]. As expected, the occurrence of dehydrin clones was high in the CA library. Somewhat surprisingly, dehydrin clones were picked at a comparatively high frequency from the NA library (nine times as compared to 10 times from the CA library). By Northern blot analysis, dehydrin message levels have been shown to be relatively low in blueberry floral buds before cold acclimation and to rise markedly during cold acclimation [15]. The apparent discrepency between these results could be explained if dehydrin message levels, although low in NA buds relative to CA ones, are still high relative to other messages present in NA buds.

Clones homologous to an early light-inducible protein, beta amylase, and Mei2-like cell cycle protein were each picked four times from the CA library and not at all from the NA library (Table 2), suggesting that they, too, might represent cold acclimation-responsive transcripts. Early light-inducible proteins are nuclear-encoded thylakoid membrane proteins related to light-harvesting chlorophyll a/b binding (Cab) proteins [40,41]. They are absent in dark-grown seedlings and their message levels increase transiently during early stages of maturation of etioplasts to chloroplasts [42,43]. They are believed to be involved in protecting photosystem II against light stress [44,45]. CA floral buds of blueberry collected during mid-December would have experienced shorter photoperiods than NA ones, collected at the first of October. Thus, photoperiod rather than cold stress could explain the abundance of this clone in the CA library. Similarly, the gene encoding Early Light-Induced Protein 1 was found to be one of the most rapid and highly induced genes in cold-stressed *Arabidopsis* plants. However, these plants were also grown under reduced light intensity as compared to control plants [13]. Therefore, whether this gene is induced under cold stress, in the absence of light stress, remains to be determined. The message encoding the starch-degrading enzyme, beta amylase, is also up-regulated during cold stress in *Arabidopsis* [12,13]. Starch accumulates in the fall in buds of several tree species and then disappears during the winter [46,47]. Beta amylase could be involved in breaking down starch in buds to form other carbohydrates that act as osmoprotectants and increase freezing tolerance. Mei2 is a protein involved in the regulation of meiosis [48,49]. To date, it has not been described as being cold-responsive, but cold-stress microarray experiments in *Arabidopsis* have not focused on gene expression in meiotic floral tissue or whole floral buds, as was done in this study. Furthermore, three additional cDNAs with similarity to protein sequences in Genbank of unknown function were picked four to five times each from the CA library and not at all from the NA library.

Several cDNAs were highly abundant in the NA library and not in the CA library (Table 3), suggesting that they may represent transcripts that are down-regulated with

Table 3
Most abundant cDNAs from the non-acclimated library

Putative gene identification	# cDNAs[a]
Dehydrin	9 (10 in CA)[b]
Histone H3.2 protein	7 (0 in CA)
Metallothionein-like protein Type 2	7 (1 in CA)
BURP-domain dehydration-responsive protein RD 22	6 (1 in CA)
F1 ATPase subunit alpha	5 (5 in CA)
Glyceraldehyde-3-phosphate dehydrogenase	4 (1 in CA)

[a] Number of times a particular cDNA was picked from the NA library.
[b] In parentheses is the number of times a particular cDNA was picked from the CA library for comparison.

cold acclimation. These include cDNAs encoding histone H3.2, metallothionein-like protein Type 2, BURP-domain dehydration-responsive protein RD 22, and glyceraldehyde-3-phosphate dehydrogenase. Although none of these genes were identified as being down-regulated with cold stress from microarray studies in *Arabidopsis* [13], the expression patterns of some of them in other woody plants are consistent with their higher abundance in the NA library. For example, activity of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, has been shown to be much lower in dormant apple leaf buds than in nondormant buds [50]. Blueberry floral buds collected in mid-December would be in a deeper state of dormancy than those collected at the first of October. The BURP-domain protein RD 22 is induced in drought and salt-stressed *Arabidopsis* plants but not in cold-stressed plants, and its induction is mediated by ABA [51]. In buds of some woody perennials, ABA levels are high and water content levels are low in the fall [47]; ABA levels then tend to decline during the cold winter months [52]. Thus, changes in ABA or water content in buds may be influencing the abundance of RD 22-transcripts in the blueberry cDNA libraries. Finally, metallothionein proteins are induced in plants under a variety of stress and developmental conditions, and ESTs encoding metallothionein proteins have been found to be highly abundant in a cDNA library prepared from autumn leaves of aspen (*Populus tremula*) [53].

We have also observed differences in the occurrence of certain classes of related cDNAs between the two libraries. These classes are comprised of cDNAs that fall within multiple contigs, thus have limited homology to each other, but have similar or related functions. For example, cDNAs encoding various ubiquitin-associated proteins were picked from the NA library 26 times and only 11 times from the CA library. Within this class of cDNAs was a subcategory of cDNAs encoding F-box proteins, proteins that recruit targets for ubiquitination [54,55]. Members of this subclass were picked from the NA library nine times and not at all from the CA library. The higher abundance of cDNAs encoding proteins involved in ubiquitin-dependent proteolysis in the NA library suggests that this form of proteolysis might be suspended to some degree in CA buds. Another class of related cDNAs, encoding RNA helicases, was picked from the CA library nine times and from the NA library only twice. Some RNA helicases, specifically DEAD box RNA helicases, have been shown to be up-regulated during cold stress in *Arabidopsis* [12,13]. DEAD box RNA helicases are involved in a number of different molecular mechanisms including RNA splicing, ribosome assembly, and initiation of translation [56].

3.5. Classification of ESTs with putative functions

ESTs that could be identified based on BLASTX or BLASTN search results (57% from both libraries) were classified into 14 functional groups, and then subgroups within those groups, primarily according to the catalog established for grape, *V. vinifera* [33]. The percentage of cDNAs that fell within each group for each of the libraries is shown in Table 4. The functional group with the highest percentage of cDNAs in both libraries was disease/defense (9.85% for the CA library and 8.85% for the NA library). This group includes cDNAs that are involved in disease resistance and defense and also cDNAs involved in aging/cell death, stress responses, and detoxification related to oxidative damage and binding of heavy metals. There were two subcategories, aging/cell death (11.30) and heavy metals (11.60), in which the percentage of cDNAs varied by three-fold or more between the two libraries. The higher percentage of transcripts involved in aging/cell death in the CA library resulted from picking the senescence-associated cDNA clones 10 times from that library and none from the other library. Likewise, the higher percentage of cDNAs involved in binding heavy metals in the NA library was the result of picking metallothionein cDNAs seven times from that library and only once from the other library.

The class of cDNAs involved in primary or secondary metabolism (9.36% from the CA library and 7.66% from the NA library) was also highly represented. Within the secondary metabolism category, it is interesting to note that there was more than a three-fold higher percentage of cDNAs involved in phenylpropanoid/phenolic metabolism (12.10) from the NA library than from the CA library. This is consistent with the observation that blueberry plants accumulate anthocyanins in the fall in response to cooler temperatures, and turn bright red [57].

Some of the other major differences (three-fold or greater) between the two libraries, in terms of percentage of cDNAs within the various subcategories, can be explained by the highly abundant cDNAs that have already been discussed. The higher percentage of cDNAs involved in chloroplast biogenesis (09.40) in the CA library results from the abundance of cDNAs encoding the early-light-inducible proteins and other chlorophyll a/b-binding proteins. The higher percentage of cDNAs involved in chromatin modification (04.40) in the NA library was due to the higher abundance of cDNAs encoding the histone H3.2 protein. In addition to these, there were more than three-fold higher percentages of cDNAs in the NA library encoding proteins involved in protein modification (06.20), receptors involved in signal transduction (10.10), and proteins involved in general fruit and flower development (13.20). In the CA library, there were higher percentages of cDNAs encoding putative oxygenases (01.80—general/other subcategory under primary metabolism category), proteins involved in the TCA pathway (02.30), putative transposases (03.30—general/other subcategory under cell growth/division category), and ion transporters (07.10). It is interesting to speculate that the higher percentage of cDNAs encoding putative transposases in the CA library may be a reflection of higher activity of transposons under the stressful conditions of winter. On

Table 4
Percentage of cDNAs in each functional category for the cold acclimated (CA) and non-acclimated (NA) libraries

Functional category	CA (%)	NA (%)
01 Primary metabolism	*8.71*	*5.79*
01.10 Amino acid	1.81	1.36
01.20 Nitrogen and sulfur	0.16	0.34
01.30 Nucleotides	0.82	0.34
01.40 Phosphate	0.33	0.34
01.50 Sugars and polysaccharides	3.12	2.39
01.60 Lipids and sterols	1.15	0.68
01.70 Cofactors	0.66	0.34
01.80 General/other	0.66*[a]	0.00
02 Energy	*5.42*	*4.59*
02.10 Glycolysis	1.48	1.19
02.20 Gluconeogenesis	0.33	0.00
02.30 TCA pathway	0.82*	0.17
02.40 Electron transport/respiration and photosynthesis	1.48	1.70
02.50 Calvin cycle	0.16	0.34
02.60 General/other	1.15	1.19
03 Cell growth/division	*1.97*	*1.02*
03.10 DNA synthesis/replication	0.00	0.34
03.20 Cell cycle	0.82	0.34
03.30 General/other	1.15*	0.34
04 Transcription	*4.92*	*5.78*
04.10 RNA synthesis	1.15	1.19
04.20 General TFs	0.66	1.36
04.30 Unknown DNA-binding protein	0.49	0.34
04.40 Chromatin modification	0.49	1.70*
04.50 mRNA processing	2.13	1.19
05 Protein synthesis	*4.93*	*5.28*
05.10 Ribosomal proteins	2.79	3.07
05.20 Translation factors	1.15	1.19
05.30 Translational control/RNA-binding proteins	0.66	0.51
05.40 tRNA synthases	0.33	0.51
06 Protein destination and storage	*4.76*	*6.81*
06.10 Folding and stability	1.15	0.85
06.20 Modification	0.00	0.68*
06.30 Proteolysis	3.61	5.28
07 Transporters	*2.79*	*2.55*
07.10 Ions	0.66*	0.17
07.20 Sugars	0.33	0.34
07.30 Amino acids	0.00	0.17
07.40 Lipids	0.49	0.51
07.50 ATPase	0.00	0.17
07.60 ABC-type	0.82	0.85
07.70 General/other	0.49	0.34
08 Intracellular traffic	*0.82*	*1.02*
08.10 Mitochondrial	0.00	0.17
08.20 Vesicular	0.66	0.85
08.30 General/other	0.16	0.00
09 Cell structure	*4.43*	*3.06*
09.10 Cell wall	1.97	1.53
09.20 Cytoskeleton	0.82	1.19
09.30 ER/Golgi	0.16	0.00
09.40 Chloroplast	1.48*	0.34
10 Signal transduction	*3.93*	*6.46*
10.10 Receptors	0.00	1.02*
10.20 Mediators	0.82	1.36
10.30 Kinases	2.13	2.21
10.40 Phosphatases	0.49	0.68
10.50 G proteins	0.49	1.19
11 Disease/defense	*9.85*	*8.85*
11.10 Resistance genes	0.00	0.17
11.20 Defense-regulated	0.66	1.36
11.30 Aging/cell death	1.64*	0.00
11.40 Stress responses	5.91	4.60
11.50 Detoxification/antioxidants	1.31	1.36
11.60 Heavy metals	0.33	1.36*
12 Secondary metabolism	*0.65*	*1.87**
12.10 Phenylpropanoids/phenolics	0.49	1.70*
12.20 Amines	0.16	0.17
13 Development	*3.11*	*2.72*
13.10 Leaf and stem/general	0.00	0.17
13.11 Leaf and stem-specific TFs	0.33	0.68
13.20 Fruit and flower/general	0.16	0.68*
13.21 Fruit and flower-specific TFs	0.49	0.00
13.30 General/other	2.13	1.19
14 Hormone-related	*0.82*	*1.02*
14.10 Metabolism	0.16	0.00
14.20 Receptor	0.00	0.17
14.30 Induced	0.33	0.51
14.40 Repressed	0.33	0.34
Unclassified	*42.86*	*43.10*
Hypothetical/unknown putative proteins	27.26	26.75
No significant similarity	15.60	16.35

[a] Asterisks indicate a more than three-fold higher percentage of cDNAs in a particular category or subcategory from one library vs. the other library. If there were no cDNAs of a particular category or subcategory picked from one library (0%), then the percentage picked from the other library was compared to 0.16 or 0.17%, which is the percentage if one cDNA had been picked from the CA (1/609) or NA (1/587) library, respectively, rather than 0%, to determine if there was a three-fold difference.

the other hand, the higher percentage of cDNAs encoding TCA enzymes in the CA library is surprising in light of other findings that the activities of enzymes involved in the TCA pathway are lower in dormant buds than in buds that are resuming growth [58]. However, this previous work studied gene expression at the enzyme activity level, not at the RNA level, as was done here. Whether these genes are really induced at the RNA level under cold acclimating conditions, or whether they were simply picked more often from the CA library by chance, remains to be determined from microarray or northern analyses.

Also of interest were several cDNAs encoding specific transcription factors, related to leaf and stem (13.11) or fruit and flower (13.21) development, that were isolated from one or both libraries. These include KNOTTED-1-like, PHANTASTICA, ROUGH SHEATH 2, SCARECROW-like, TEIL, FLOWERING LOCUS T, BEL1, LEUNIG, and CONSTANS-like B-box zinc finger proteins. In addition, under the transcription category, several cDNAs encoding general transcription factors (4.1901), such as putative

MADS box, myb-related, SET-domain, and zinc finger transcription factors, were isolated from the libraries.

3.6. Northern blot analyses of putative differentially expressed genes

Northern blots were performed to examine expression of certain transcripts that were identified as likely to be differentially expressed. Eight cDNAs were selected and used as probes on blots containing total RNA extracted from Bluecrop floral buds collected at 0, 400, 800, and 1200 chill units (Fig. 1). The selected cDNA probes encoded: a dehydrin, because our previous work [15] indicated that the message should be induced during cold acclimation; a senescence-associated protein, early light-inducible protein, beta amylase, and an unknown protein, because these cDNAs were highly abundant in the CA library but not the NA library; and histone protein H3.2, metallothionein-like protein Type 2, and BURP-domain dehydration-responsive protein RD 22, because these cDNAs were highly abundant in the NA library but not the CA library.

Results indicated that all of the messages suspected to be cold acclimation-induced were indeed present at higher levels in CA buds. All the message levels appeared to peak at about 400 or 800 chill units and then to decline, with the exception of the message encoding the unknown protein, levels of which remained high through 1200 chill units. All the cDNA probes hybridized to a single RNA species except for the cDNA encoding the senescence-associated protein, which hybridized to a 4.0 kb induced message as well as to constitutively-expressed 26S rRNA. A closer examination of the clones included in this contig revealed five contaminating 26S rRNA clones, in addition to the 10 senescence-associated protein/putative monooxygenase cDNAs. From the alignment of the DNA sequences within this contig, a 38 bp region was found to be in common between

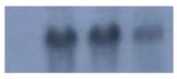
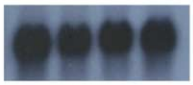
0 400 800 1200	Clone	Putative identity	Message size
	CA-36	Dehydrin	0.5 kb
	CA-500	Senescence-associated protein/ putative monooxygenase	4.0 kb
	CA-378	Early light-inducible protein	1.0 kb
	CA-482	Beta amylase	1.5 kb
	CA-265	Unknown protein	1.1 kb
	NA-1056	Histone H3.2	0.8 kb
	NA-86	Metallothionein-like protein Type 2	0.75 kb
	NA-693	BURP-domain dehydration-responsive protein RD 22	1.1 kb

Fig. 1. Northern blot analysis of expression of transcripts identified as abundant in either the CA or NA libraries. Eight cDNAs were selected and used as probes on blots of total RNA extracted from Bluecrop floral buds collected at 0, 400, 800, and 1200 chill units. Probes consisted of coding regions from genes encoding a dehydrin, senescence-associated protein, early light-inducible protein, beta amylase, protein of unknown function, histone H3.2, metallothionein-like protein Type 2, and a BURP-domain dehydration-responsive protein RD 22.

the senescence-associated protein clones and the 26S rRNA clones, explaining why the senescence-associated protein cDNA hybridized to the 26S rRNA, in addition to its own message. Hybridization to the 26S rRNA also served fortuitously as a control confirming that equal amounts of total RNA were loaded on the gels.

Northern blot analysis, using cDNA probes suspected to encode messages that are down-regulated with cold acclimation, indicated that histone H3.2 and BURP-domain dehydration-responsive protein RD 22 transcript levels did decline with cold acclimation, being significantly lower by 400 chill units. The RD 22 transcript level continued to decline through 1200 chill units, whereas histone H3.2 transcript level began to rise after its initial decline. Metallothionein Type 2 transcript level, however, remained constant throughout the chilling period, despite the fact that this cDNA was picked seven times from the NA library and only once from the CA library.

Here, we have used a genomic approach based on ESTs to study cold acclimation in blueberry with an ultimate goal of applying the information learned in developing more cold hardy cultivars. The blueberry cultivar Bluecrop was used because it is the industry standard and is fairly cold hardy. The ∼1200 5′-end ESTs (generated from ∼600 cDNA clones from each of the CA and NA libraries) and the ∼100 3′-end ESTs (generated from cDNA clones from the CA library) comprise the first publicly available EST database for blueberry. More than half of the cDNAs from which the ESTs were derived (57%) were identified based on homology to other sequences in Genbank. The identified cDNA clones have been categorized according to putative functions and comparison of the types of genes expressed under non-acclimating and cold acclimating conditions has revealed similarities to what has been found in *Arabidopsis*, as well as the potential involvement of additional types of proteins in cold acclimation in woody perennials. With the exception of metallothionein, several highly abundant cDNAs that were picked more often from one library than the other were confirmed to be differentially expressed under non-acclimating and cold acclimating conditions by Northern blot analysis. Future efforts will focus on studying patterns of expression of the set of unique blueberry cDNAs using microarray technology.

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